Influence of Punctures, Cuts, and Surface Morphologies of Golden Delicious Apples on Penetration and Growth of *Escherichia coli* O157:H7

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ABSTRACT

The ability of *Escherichia coli* O157:H7 to penetrate and grow within punctures, fresh-cut surfaces, and calyces of Golden Delicious apples was investigated. A three-strain cocktail of *E. coli* O157:H7 resistant to ampicillin was used to inoculate fresh and 48-h-old punctures, fresh-cut surfaces, and open or closed calyces. A concentric cutting procedure was used to evaluate depth of penetration within punctures and prevent cross contamination during sampling. Within 2 h, *E. coli* O157: H7 penetrated vertically through the fresh punctures and 3.4 mm within the underlying parenchyma. After 48 h, *E. coli* O157: H7 cells penetrated up to 5.5 mm within the punctures and >2.6 mm horizontally away from fresh punctures. However, 48-h-old punctures did not permit penetration beyond their boundaries. Fresh-cut surfaces permitted up to 2.8 mm penetration after 24 h. Onset of growth of *E. coli* O157:H7 occurred 4 to 8 h postinoculation on fresh punctures and fresh-cut surfaces with populations increasing by 3 logs after 48 h. *E. coli* O157:H7 penetrated within calyces regardless of the extent of opening or method of inoculation. However, *E. coli* O157:H7 was never recovered from the inner core of apples. Computed tomography scan imaging revealed that closed calyces effectively prevented penetration of sodium iodide solutions within the calyx cavity. Lack of solution penetration may explain why sanitizing treatments are ineffective in inactivating microbial cells within the calyx. Understanding the role of morphological differences in permitting or restricting bacterial penetration may lead to development of more effective strategies to enhance the safety of fresh horticultural products.

Recent reported increases in the frequency of outbreaks of foodborne illness associated with fruits and vegetables have prompted researchers to seek more effective methods to decontaminate produce *(2)*. Among the difficulties encountered is the potential for human pathogens to enter produce through natural openings as well as punctures and cuts *(6, 13)*. External barriers, such as peel, rind, and skin, play a critical role in preventing microorganisms from entering fruits. However, breaks in these barriers due to punctures caused by stems or nails during handling, or cuts in the case of fresh-cut fruits, allow pathogens such as *Escherichia coli* O157:H7 to enter and potentially grow *(10, 15)*.

The ability of *E. coli* O157:H7 to survive and grow on and within the internal structures of apple fruits and other vegetables has been documented *(9, 10, 15)*. Dingman *(10)* stressed the importance of distinguishing between surface contamination and internalization and growth when investigating the source of this organism on apples. Bacterial cells closer to the surface of produce are more likely to be affected by the action of sanitizers than cells that have been internalized. Therefore, an important question when evaluating the fate of *E. coli* O157:H7 before and after anti-microbial treatment within various regions of the apple is the concentration of this organism at various depths within apple tissue.

When evaluating movement of *E. coli* O157:H7 within apple tissues, it is important to consider the role of apple surface morphology. Buchanan et al. *(5)* attributed accumulation of dye within the inner core of Golden Delicious apples to open channels in the calyx region. They also suggested that most of the *E. coli* O157:H7 population recovered from the inner core of the calyx was due to translocation of the cells from the surface of the apples during sample preparation. Beuchat et al. *(4)* reported that there is a need to develop better methods to prepare and analyze samples to evaluate growth and survival of *E. coli* O157: H7 and other pathogens on and in various depths within produce. Therefore, the present study was conducted to determine the extent of penetration and growth of *E. coli* O157:H7 within punctured and fresh-cut apple surfaces. Another purpose was to determine the influence of open versus closed calyx morphology on penetration of *E. coli* O157:H7.

MATERIALS AND METHODS

Apples. Unwaxed Golden Delicious apples were purchased at maturity from a fruit farm in the State College, Pa., area. The apples were uniform in size and shape and did not have any vis-

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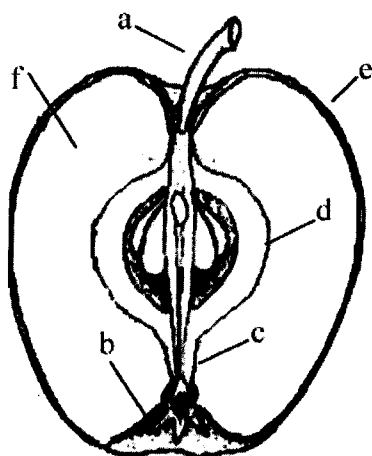
FIGURE 1. *Cross section of a Golden Delicious apple showing the stem and the stem region (a), calyx basin including the sepals (b), calyx tube (c), the inner core including carpel and seeds (d), skin (e), and the parenchyma (f).*

ible defects, cuts, or bruises. The apples were stored at 2°C until used.

Inoculum preparation. A three-strain cocktail of *E. coli* O157:H7 (strain 9727, a human isolate associated with a cider outbreak; strain 4329, a cider isolate associated with a cider outbreak; and strain 93-033, a ground beef isolate associated with a ground beef outbreak) was used throughout the study. Spontaneous mutants of *E. coli* O157:H7 resistant to 100 µg/ml ampicillin were selected as previously described *(7)*. The use of cells resistant to ampicillin facilitated enumeration of the pathogen from apple homogenate on tryptic soy agar with yeast extract (pH 7.1; Difco, BBL, Becton Dickinson, Sparks, Md.) supplemented with 100 µg/ml ampicillin (TSAYEA; Shelton Scientific, Shelton, Conn.). Most naturally occurring organisms in these samples would not be expected to grow on TSAYEA. Furthermore, use of TSAYEA allowed recovery of injured as well as noninjured *E. coli* O157:H7 cells (data not shown), thereby reducing the chance of underreporting the total population due to injury. Each pure culture was stored at −86°C in tryptic soy broth (TSB) with yeast extract supplemented with 15% glycerol. Cultures were maintained at 4°C on TSAYEA and were streaked onto fresh plates on a weekly basis.

E. coli O157:H7 was grown in tryptic soy broth with 0.6% yeast extract (Difco, BBL) supplemented with 100 µg/ml ampicillin (TSBYEA) and incubated at 37°C for 18 ± 2 h. Cells were transferred on 2 consecutive days to fresh TSBYEA prior to inoculation of apples. A portion (7 ml) of each 18-h culture was combined and centrifuged at 10 × *g* and 4°C for 10 min. Cells were washed twice in 0.85% saline and resuspended in 0.85% saline. Cells were then serially diluted in buffered peptone water (BPW; Difco, BBL, Becton Dickinson) to achieve the desired concentrations prior to inoculation of apples.

Inoculation of apples. Figure 1 shows a diagram of the apple regions analyzed in this study. A set of three apples at 21°C was punctured at the equator halfway between the apex and the base with a sterile finishing nail (2.6 mm wide by 5 mm deep) to simulate mechanical injury that may occur during harvest or postharvest handling. Apples were spot inoculated with 25 µl (4.5 to 4.9 log CFU) of the inoculum placed dropwise on the outside of the puncture. Following inoculation, the surfaces of apples outside of an 18.8-mm radius surrounding the puncture area were wiped

with 70% ethanol, and the apples were allowed to dry at 21°C for various times. Experiments were also performed at 7°C (data not shown).

For 48-h-old punctures, apples were punctured as described above but were allowed to remain at 7°C for 48 h to allow time for the puncture to dry. After drying, apples were tempered to 21°C and inoculated as previously described.

Fresh-cut surfaces were prepared by first removing an apple plug from the equator of the apple. The skin was removed with a sterile surgical blade, and the plug was cut into three pieces of approximately 7 mm each with a sharp kitchen knife with a straight sterile blade. A set of three apple disks was placed in a sterile petri dish, and 25 µl of the inoculum was placed dropwise on the apple disks. The inoculated disks were incubated at 7°C or 21°C for various times. All cutting equipment used throughout the study was sterilized by autoclaving at 121°C for 15 min.

Calyces were visually categorized as open or closed based on the degree of sepal covering in the calyx cavity of the fruit. If the sepals completely covered the cavity, they were categorized as closed. If they remained upright or partly upright, they were categorized as open. Calyx regions were spot inoculated as described for puncture with the exception that the inoculum was increased to 100 µl in order to simulate the quantity of liquid that may accumulate on the calyx in the field from spray irrigation or rain or following a postharvest rinse. For dip inoculation, apples at 22°C were mounted on sterile three-prong extension clamps attached to an adjustable angle clamp (Fisher Scientific, Pittsburgh, Pa.) and then submerged with the calyx opening parallel with the ground and held in 500 ml of inoculum at 7°C for 5 min. Solutions were agitated with 51-mm octagonal magnetic stir bars (Fisher Scientific) spinning at 50 rpm. Apples were incubated for various times in a class II type A/B3 biosafety hood (SterilGard Hood, The Baker Company, Inc. Sanford, Mass.) at 21°C. Dip inoculation also was performed with no temperature difference between the apple and inoculum (data not shown).

Evaluation of bacterial penetration. To evaluate penetration within punctures, two sterile concentric stainless steel cork borers (8.0 and 18.8 mm inside diameter) were mounted on a drill press as shown in Figure 2A. The two cork borers were aligned with a sterile apple holder on the drill stage by using a laser pointer mounted below the stage (Fig. 2A). Following incubation, apples were cut in half perpendicular to the direction of the puncture. One apple half was placed with the puncture facing down through the opening of the apple holder so that the uninoculated side was facing the cork borers (Fig. 2B). Once the apple half was centered on the stage, the cork borers were lowered onto the apple, which was then cut from the area of no inoculation to the area of high inoculation. This process resulted in two concentric (inner and outer) apple plugs. To prevent cross contamination, each apple plug was then removed from the opposite end of the cork borer and placed on a sterile cutting board. The plugs were then cut at 2.1-mm increments with a set of sterile Double Edge razor blades (Schick, Milford, Conn.) fixed on a set of threaded rods and separated from each other with three 0.7-mm washers (Fig. 2C and 2D). Evaluation of penetration of the cells was also conducted at 7°C to confirm that recovery of cells at greater depths was due to bacterial penetration within the fresh-cut surface and not due to cross contamination as a result of high bacterial growth.

To evaluate penetration within fresh-cut surfaces, inoculated apple disks were sectioned with the razor blades as described above, except that the blades were spaced 1.4 mm apart. Thinner
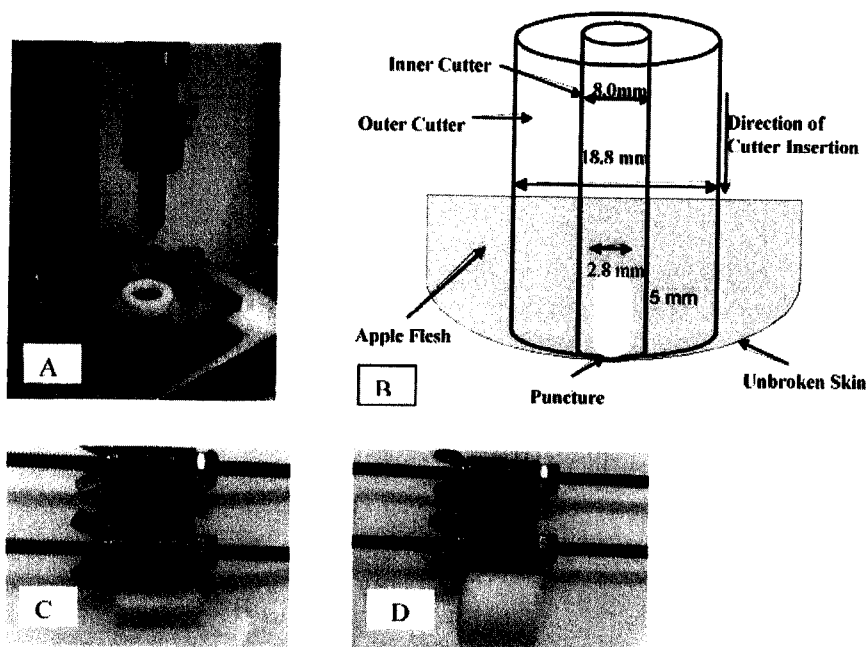
FIGURE 2. *Cutting procedure used in this study showing the concentric cork borers (A), direction of cutter insertion and their distance from the puncture (B), and the set of fixed razor blades used to cut the inner (C) and the outer (D) sections surrounding the puncture hole.*

sections allowed better examination of the extent of penetration of the *E. coli* O157:H7 within the cut surface.

The cross section of a Golden Delicious apple depicting the calyx basin, sepals, calyx cavity, carpel, and seeds is shown in Figure 1. The inoculated apple was cut in half at its equator so that one half contained the calyx. The skin just above the calyx basin was removed with a flame-sterilized knife as described by Annous et al. *(1)* in order to prevent carryover of the *E. coli* O157: H7 cells from skin to the inner cortex during sectioning. A sterile cork borer (19 mm inner diameter) was used to excise an apple plug containing the calyx and the calyx tube extending to the core flesh and part of the carpel and seeds. The apple plug was placed on a sterile cutting board and sectioned with the razor-blade cutting mechanism described above, except that the blades were spaced approximately 3 mm apart. Thicker sections were cut because the hollow and conical shape of these regions required larger pieces to withstand the cutting pressure. The cutters were positioned so that the lowest portion of the calyx basin and any protruding sepals were included in the first (basin) section. This allowed for enumeration of *E. coli* O157:H7 cells outside the calyx cavity. Therefore, the subsequent sections included depths of 0 to 3 mm, 3.1 to 6 mm, and so on up to 15 mm within the calyx cavity. Each resulting apple disk was aseptically transferred to a separate 120-ml stomacher bag (Nasco Whirl-Pak, Fort Atkinson, Wis.). Corresponding sections from two other apples were combined in separate stomacher bags for each experiment. A set of three samples from each corresponding section from various regions of the apple were pooled, weighed, and diluted with sufficient $2\times$ BPW to achieve a 1:10 dilution. The samples were then homogenized for 2 min at low speed in a model 80 stomacher (Seward Ltd., Thetford, Norfolk, UK) and decimally diluted with $1\times$ BPW, plated on TSAYEA, and incubated at 37°C for 24 ± 2 h.

After samples of blended apple pieces were removed from the stomacher bag for enumeration on TSAYEA, the remaining $2\times$ BPW solution from each cut section was added to an equal volume of double-strength TSBYEA and incubated at 37°C for 24 ± 2 h. This was a modification of the enrichment step described by Kenney and Beuchat *(16)*, who used $2\times$ modified TSB. If *E. coli* O157:H7 did not form colonies on the TSAYEA plates, a sample of the enrichment broth was then streaked onto TSAYEA

and incubated at 37°C for 24 ± 2 h. Colonies on TSAYEA were confirmed as *E. coli* O157:H7 with the O157 latex agglutination assay (Oxoid, Basingstoke, Hampshire, UK).

CT scan imaging of fluid movement within apples. Sodium iodide (NaI) and a third-generation industrial X-ray computed tomography (CT) scanner (OMNI-X, Universal HD-600, Universal Systems, Solon, Ohio) were used to measure the effect of punctures, differences in apple surface morphology, and temperature differential on movement of treatment solutions within apples. With this type of scanner, the X-ray source and detector system are fixed while the sample is rotated at a specified position between source and detector to obtain the X-ray projections at the desired image magnification. Apples at 22°C were fixed horizontally with mounting putty (Manco, Inc., Avon, Ohio) in a polyvinyl chloride container 4.5 in. (11.43 cm) in diameter. A circular handle at the bottom of the container was held in place by the chucks of the sample-positioning table. Approximately 700 ml of a 2% NaI tracer solution was added to the container, completely covering the apple. NaI solution was used to create contrast between the liquid phase and the apple flesh and thus enhanced measurement of treatment penetration.

A micro-focus X-ray tube, capable of providing X rays (225 kV [maximum], 1 mA [maximum]) was used. To obtain optimum resolution and contrast, the X-ray intensity during the scans was set at 130 kV and 0.5 mA. The position of the samples was set to obtain 77 μm in each pixel when the whole diameter of the apple could be viewed. The detector system was a 25-cm-diameter area detector capable of collecting up to 100 scans during a single rotation. At this magnification, image thicknesses were 84 μm. Image quantification was performed by VoxelCalc, operating under UNIX environment. The CT-number images presented were colored in such a way that light colors (yellow and orange) represented high CT numbers (high density and atomic number); colors such as red had a lower CT number and blue had the lowest CT number, which represented air and air-filled portions of the interior of the apple, respectively.

Different scenarios were tested to investigate the fluid movement within apples. Fluid movements within open or closed apple calyces, 2.6 by 5 mm punctures, and within the stem region (not

presented) were evaluated under negative temperature differential (apple, 23 to 25°C; solution, 7 to 10°C). Also, the effect of low pressure (5 lb/in^2) in enhancing fluid penetration and movement within these regions was evaluated. Scanning and image processing were performed at four to five different times in order to observe the temporal and spatial variations of fluid movement within the apple. The images obtained from the detector system were reconstructed in a 1,024 by 1,024 pixel matrix.

Statistical analysis. All experiments were performed at least in triplicate, and at least two samples were analyzed at each sample time. Analysis of variance and Duncan multiple range tests (Minitab Corp., State College, Pa.) were performed to determine significant differences ($P \leq 0.05$) in log CFU between treatments.

RESULTS

Penetration and growth of *E. coli* O157:H7 within fresh punctures and fresh-cut surfaces. Penetration of inocula was observed within the puncture openings and through damaged apple parenchyma cells and cuts (Fig. 3A through 3C). Downward or vertical penetration of the inocula was observed over time. Within the first 2 h, *E. coli* O157:H7 cells penetrated through the 5-mm-deep punctures and up to 3.4 mm vertically within the underlying parenchyma (Fig. 4). The majority (95%) of the *E. coli* O157:H7 cells recovered were from the first two sections (0 to 2.1 and 2.2 to 4.2 mm). The remaining population of *E. coli* O157:H7 resided within depths of 4.3 to 8.4 mm (Fig. 3A). Horizontal penetration (away from the walls of the punctures) remained within the inner region after 2 h without any recovery in the outer region. *E. coli* O157:H7 was not detected at depths of 8.5 mm or higher after 2 h by the enrichment procedure described above. *E. coli* O157:H7 cells were found at greater depths (up to 10.5 mm) vertically within the puncture after 48 h, indicating movement of cells within apple tissue over time (Fig. 3C). Incubation of the apples at 7°C followed by enrichment resulted in recovery of the cells from similar depths (data not shown). Horizontal penetration (>2.6 mm) of the cells was observed at the 0- to 2.1-mm depth interval after 48 h at 21°C (Fig. 3B). No other cells were recovered at other outer depths. The concentration of *E. coli* O157:H7 at outer regions was significantly lower than the concentration within the corresponding inner region ($P < 0.05$). However, repeated recovery of cells at horizontal distances greater than 2.6 mm away from the boundaries of the punctures indicated horizontal movement of the cells over time.

No significant growth was observed at 21°C after 2 h post inoculation (Fig. 5). Onset of growth of *E. coli* O157:H7 at 21°C occurred between 4 to 8 h postinoculation (Fig. 4). Maximum growth of *E. coli* O157:H7 (2.57 log CFU/g increase) was observed within the inner region of the puncture after 48 h at 21°C (Fig. 3A). Growth was observed at all depth intervals; however, the majority of the growth occurred within the 0- to 2.1-mm section (2.86 log) and the 2.2- to 4.2-mm section (3.21 log) inside the punctures (Fig. 3A). The 3.21 log CFU/g increase in the 2.2- to 4.2-mm section accounted for the largest margin of increase among all sections studied.

Movement of the inoculum within fresh cut apples was

limited to the top 1.4-mm section of the apples after 8 h (Fig. 4). However, *E. coli* O157:H7 was detected within the 1.5- to 2.8-mm section after 24 h at 21°C. This trend continued up to 144 h postinoculation without further penetration.

Onset of growth within fresh-cut surfaces occurred between 4 and 8 h postinoculation at 21°C (Fig. 5). *E. coli* O157:H7 concentrations reached maximum levels after 96 h at 21°C. *E. coli* O157:H7 was able to survive without significant change at 7°C (data not shown). Penetration of the cells to the 1.5- to 2.8-mm section within fresh-cut surfaces was also observed at 7°C with enrichment (data not shown).

Penetration and growth of *E. coli* O157:H7 within 48-h-old punctures. Penetration of *E. coli* O157:H7 within 48-h-old punctures was limited to within the first two sections (Fig. 3B). Horizontal penetration of the inoculum remained within the inner region. There were no significant differences between the concentrations of cells at either section. No significant growth was observed at 21°C between 2 and 4 h postinoculation (data not shown). After 48 h, there was a 0.77 log CFU/g increase in the 0- to 2.1-mm section. The majority of growth within the puncture occurred in the 2.2- to 4.2-mm section (1.98 log CFU/g increase).

Penetration of *E. coli* O157:H7 within open and closed calyces following dip or spot inoculation. Table 1 shows penetration following a 5-min dip inoculation under negative temperature differential (apple 21°C, inoculum 4°C). Open calyces allowed penetration up to 15.0 mm, whereas closed calyces allowed penetration up to 9.0 mm. Cells were found to have penetrated up to 9.0 mm within the open calyces; however, enrichment revealed the presence of *E. coli* O157:H7 at depths up to 15 mm. Similarly, cells were found to have penetrated up to 6.0 mm within the closed calyces; however, the enrichment procedure revealed the presence of *E. coli* O157:H7 at depths up to 9.0 mm. The number of cells on the basin of the closed calyces was significantly lower than the next two sections ($P < 0.05$). There was no significant difference between the number of cells recovered from the basin and up to 9.0 mm within the open calyces ($P > 0.05$). *E. coli* O157:H7 was not recovered from the ventral cavity or the seed locules at any point throughout the study.

Table 2 shows penetration of *E. coli* O157:H7 within open and closed calyces following spot inoculation with 100 µl (approximately 4.8 log CFU) when the apple was at 21°C and the inoculum was at 4°C. Open calyces allowed penetration up to 12 mm, whereas penetration of the cells within a closed calyx was limited to 9.0 mm. The majority of cells appeared to have penetrated within the open calyx cavity, for a significantly higher number of cells ($P < 0.05$) were recovered from the 0- to 9.0-mm sections than from the basin. The majority of cells on the closed calyx were limited to the basin and up to 3.0 mm within the cavity (Table 2). Experiments conducted with no temperature difference between the apple and inoculum led to similar results (data not shown).
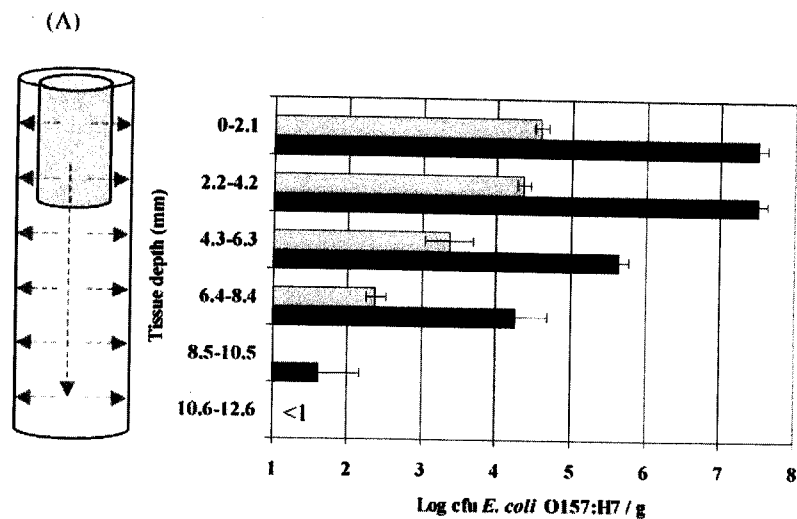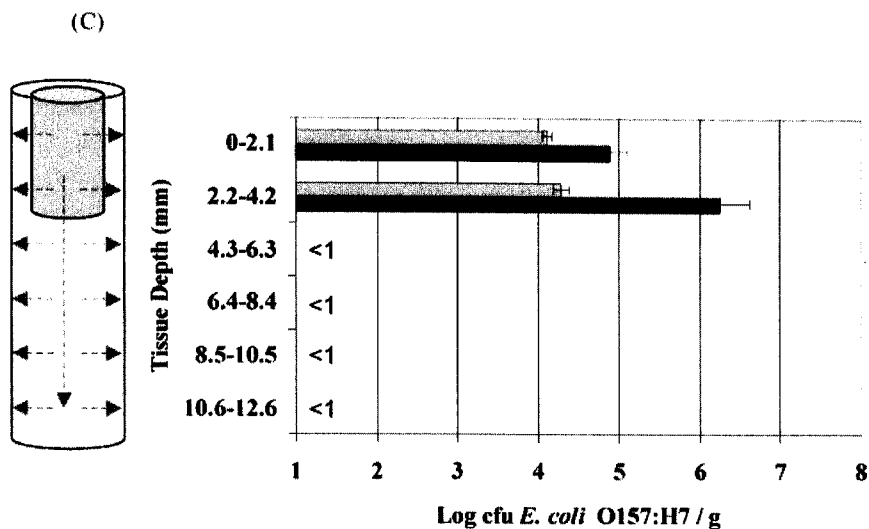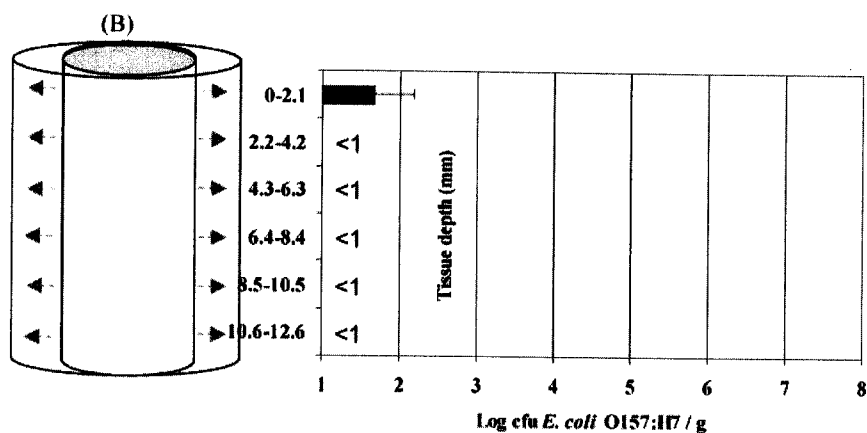
(A)

FIGURE 3. *Penetration and growth of* E. coli *O157:H7 within and through the inner (A) and outer regions (B) of the fresh and 48-h old (C) punctures after 2 h (gray) and 48 h (black)* (P < 0.05).

(B)

(C)

CT scan imaging of fluid penetration within apples. CT scan imaging of fluid movement within open and closed calyces and within punctures are shown in Figure 6A through 6D. NaI entered the open calyx cavity and reached the interior filament and style remnants within 4 min (Fig. 6A1). After 21 min, the solution filled the calyx cavity and reached the outer limits of the ovary wall (Fig. 6A2). How-

ever, the solution did not appear to penetrate within the ventral cavity and seed locules. The flower remnants appeared to absorb the solution as they became increasingly difficult to discern over time. Movement of the solution within the closed calyx was not as pronounced (Fig. 6B1). The closed calyx prevented penetration of solution within the first 2 min of submersion. Submersion for an additional
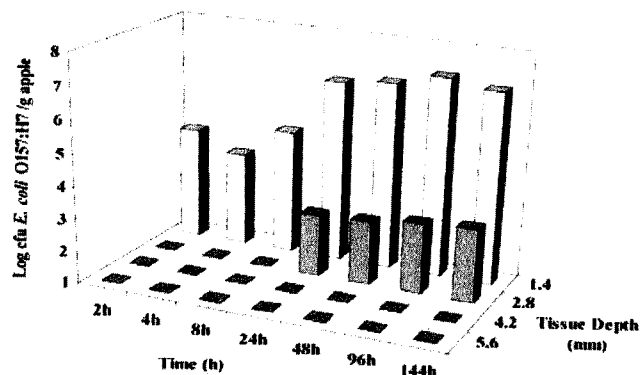
FIGURE 4. *Penetration and growth of* E. coli *O157:H7 within fresh-cut surfaces at various depths over time (P < 0.05).*

15 and 29 min showed little further penetration along the length of the calyx cavity (Fig. 6B2 and 6B3).

CT scan images of fluid movement within a fresh puncture near the stem end are shown in Figure 6C1 through 6C3. Within 3 min, fluid had entered the puncture (2.6 by 5 mm). However, penetration of NaI solution within the underlying parenchyma was not discernable. Freshly removed stem scar did not permit penetration of the solution to the ventral cavity after 22 min (data not shown). Although the fresh scar tissue appeared to absorb the solution, it did not permit penetration of NaI into the apple core.

Effect of low pressure (5 lb/in^2) on infiltration of the NaI tracer solution within a closed calyx and intact apple skin was evaluated (Fig. 6D1 through 6D3). As stated previously, closed calyces were effective barriers against penetration of the solution within the calyx cavity. However, pressurization for 15 min caused the solution to fill the cavity up to the outer limits of the ovary wall. Pressurization for another 12 min did not result in further infiltration of the solution. The bright area within the seed locules and ventral cavity of the apples after 15 and 27 min of pressurization was the result of a shift in apple size and position due to pressure and not fluid movement into this area. This was confirmed by comparing the image intensities from this region and that of the NaI solution. Pressurization resulted in penetration of the solution within the cuticular region and into apple tissues. The equator (widest point between
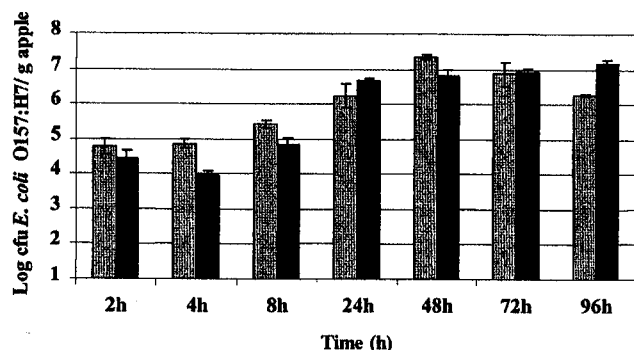

FIGURE 5. *Growth of* E. coli *O157:H7 within fresh punctures (gray) and fresh cut surfaces (black) held at 21°C for up to 96 h (P < 0.05).*

TABLE 1. *Penetration of* E. coli *O157:H7 within open and closed apple calyces after 5-min dip inoculation*[a]

	Open calyx		Closed calyx	
	Log CFU/g	Enrichment (no. positive/ total)	Log CFU/g	Enrichment (no. positive/ total)
Basin	5.02 A[b]	ND[c]	4.00 B	ND
0–3.0	5.04 A	ND	5.20 A	ND
3.1–6.0	4.69 A	ND	5.05 A	ND
6.1–9.0	4.39 A	ND	<1	(2/4)
9.1–12.0	<1	(2/4)	<1	(0/4)
12.1–15.0	<1	(1/4)	<1	(0/4)

[a] Apple temperature was 21°C; inoculum temperature was 4°C.
[b] Values with same letters within columns are not significantly different ($P < 0.05$).
[c] ND, not done.

the apex and the base of the apple) appeared to be the area most vulnerable to pressure infiltration.

DISCUSSION

Human pathogens can enter produce via several points during preharvest, harvest, and postharvest processing *(3)*. During postharvest handling of apples, water used to remove debris, drench, convey, and wash apples can become a source of contamination. Goverd et al. *(11)* reported that flume water can be a potential source of contamination of apples. Moreover, Luedtke and Powell *(20)* reported that only 40% of the processors surveyed used sanitizer in their wash water. Buchanan et al. *(5)* reported that there is a greater potential for infiltration of bacteria within the outer core regions of the apple calyx when the water temperature is lower than that of the apple. Other researchers have also reported penetration of *E. coli* O157:H7 within natural openings on the surface *(6, 21)* as well as those caused by injury to the tissue *(6, 13, 23)*.

Avoidance of cross contamination during sample preparation when evaluating microbial contamination and inter-

TABLE 2. *Penetration of* E. coli *O157:H7 within open and closed apple calyces following spot inoculation with 100 μl of the inoculum*[a]

	Open calyx		Closed calyx	
	Log CFU/g	Enrichment (no. positive/ total)	Log CFU/g	Enrichment (no. positive/ total)
Basin	2.98 B[b]	ND[c]	4.20 A	ND
0–3.0	4.04 A	ND	3.64 A	ND
3.1–6.0	3.82 A	ND	2.58 B	ND
6.1–9.0	3.71 A	ND	<1	(1/4)
9.1–12.0	<1	(2/4)	<1	(0/4)
12.1–15.0	<1	(0/4)	<1	(0/4)

[a] Apple temperature was 21°C; inoculum temperature was 4°C.
[b] Values with same letters within columns are not significantly different ($P < 0.05$).
[c] ND, not done.

After 4 min | After 21 min

A1 | Solution f | floral tube | A2

After 2 min | After 15 min | After 29 min

B1 | Trapped a | B2 | Solution f | floral tube | B3

After 3 min | After 11 min | After 22 min

C1 | C2 | Solution fro | puncture | C3

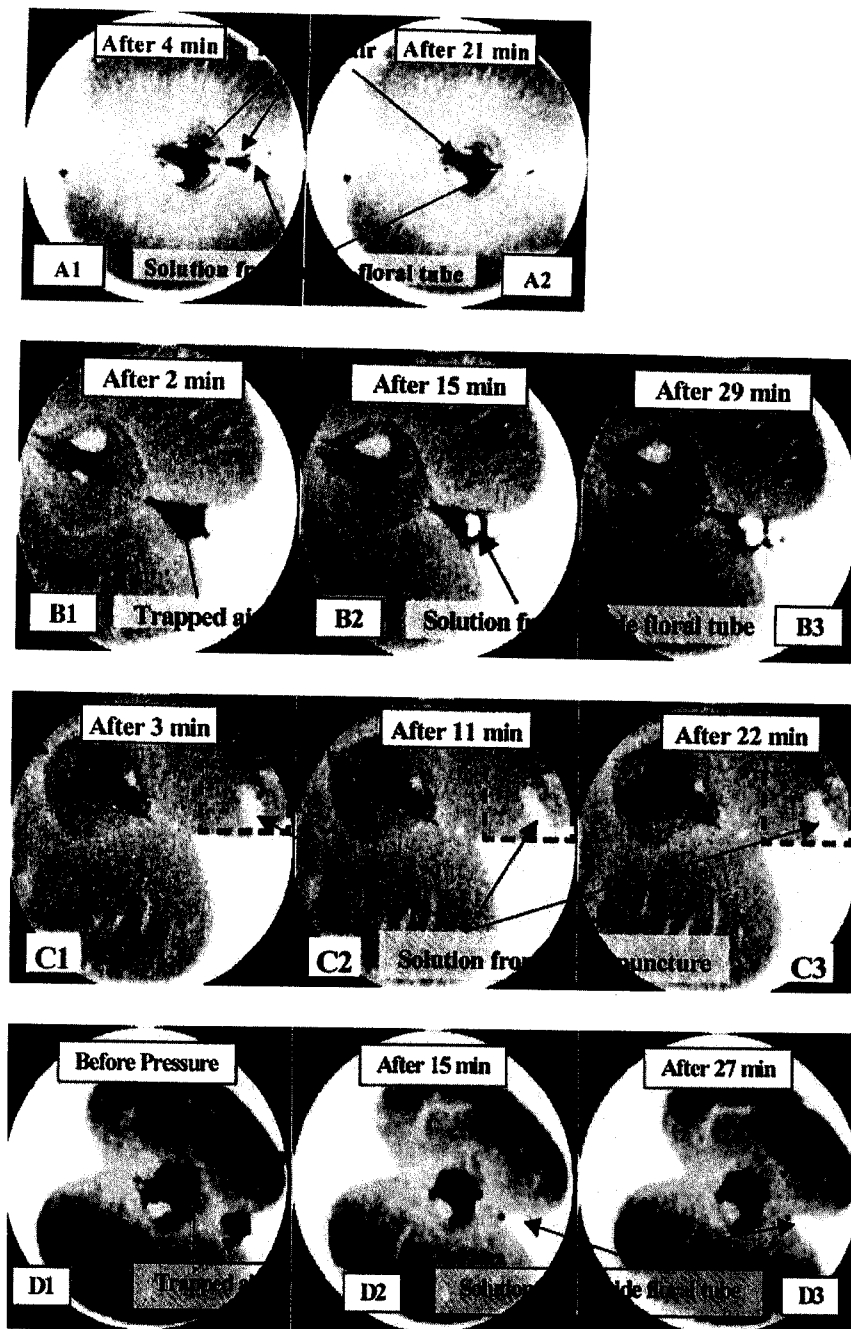Before Pressure | After 15 min | After 27 min

D1 | D2 | D3

FIGURE 6. CT scan images of penetration of NaI solution within a closed (A1 through A2) and an open (B1 through B3) apple calyx under negative temperature differential (25°C apple, 4°C solution), within a fresh apple puncture (C1 through C3), and within a closed calyx before and after pressurization with 5% lb/in^2 (D1 through D3).

nalization is necessary to obtain valid data. Buchanan et al. (5) suggested that most of the E. coli O157:H7 cells associated with the inner core of apples may have translocated from the surface during cutting. Beuchat et al. (4) pointed out the need for a standard method to accurately determine the presence and populations of pathogenic microorganisms on produce. The present study used a sample-preparation procedure to prevent cross contamination during sampling.

Our study supports evidence provided by others that E. coli O157:H7 can enter the natural openings as well as the cut surfaces of apples, where they can survive and potentially grow (10, 13–15). However, inserting the cutting equipment from the area of high contamination can result in carryover of the cells to greater depths within apples when evaluating the extent of penetration within porous tissues such as puncture or fresh-cut slices (unpublished observation). This may lead to inaccurate estimation of the extent of bacterial penetration. Janes et al. (13) reported that bacteria penetrated up to 23 mm within a 7-mm-deep apple puncture created by an artist's knife. Burnett et al. (6) reported that E. coli O157:H7 preferentially attached to damaged tissue surrounding a 10-mm puncture wound; however, they only penetrated 70 μm into the puncture hole. Stopforth et al. (23) reported penetration of E. coli O157: H7 up to 18 mm away from the apple punctures after 1 h of inoculation. Using our procedure, we demonstrated that penetration of E. coli O157:H7 within the open puncture and subsequently within the apple tissue is a gradual process and is dependent on time and the condition of the puncture. This may be explained by considering the abun-

dance of intercellular space within the deeper underlying parenchyma of mature fruit. Puncturing resulted in damage to the cuticle, epidermal, and subepidermal parenchyma. Along with the release of nutrients from the damaged cells, puncturing may have created large empty regions as a result of loosening of underlying cell-to-cell connections. This may have given the inoculum access to the intercellular space and created a route through which it could penetrate. Experiments with nails with blunt ends, simulating stem punctures, yielded similar results as puncturing with sharp points (unpublished observation).

However, 48-h-old punctures did not permit penetration beyond the boundaries of the punctures. This may have been due to lignification and drying of the punctured tissue, which prevented further moisture loss prior to inoculation. Within the fresh puncture, release of moisture and nutrients but not large amounts of inhibitory malic acid, which is located in vacuoles within the apple cells, may have permitted growth of E. coli O157:H7 to high numbers. In contrast, release of malic acid during pressing may explain the low pH of apple cider and why E. coli O157:H7 does not grow in this product. Janisiewics et al. (14) suggested that growth of E. coli O157:H7 on cut apple tissue may be a result of the ability of the bacteria to modify their microenvironment. These modifications would be more difficult to achieve in apple juice or cider. E. coli O157:H7 did not grow as well within the 48-h-old punctures. This may have been due to lack of nutrients and moisture necessary for growth. Depletion of available nutrients and space by the competing microflora has also been suggested (14). Pre- or coinoculation of apple wounds with a strain of Pseudomonas syringae has been shown to prevent growth of E. coli O157:H7 (15).

E. coli O157:H7 was able to grow within the fresh-cut surface of apples. This was similar to the findings of Gunes and Hotchkiss (12), who reported that E. coli O157:H7 grew on apple slices at 15 and 20°C. Growth of other human pathogens on apple slices has been reported. Conway et al. (8) reported growth of Listeria monocytogenes on apple slices at 10 and 20°C. Liao and Sapers (19) reported that Salmonella Chester grew on fresh-cut apples at 20°C and survived subsequent sanitizer treatments. The authors suggested that induced resistance to sanitizers due to attachment was likely the reason for survival. Burnett et al. (6) suggested that by attaching to internal tissues of apples, E. coli O157:H7 may evade decontamination treatments. In our study, we observed that cells of E. coli O157:H7 penetrated fresh-cut surfaces as well as punctured areas and began growth 4 to 8 h postinoculation at 21°C. This emphasizes the need to strictly control the temperature of the apple and the promptness of processing in order to prevent further growth of this pathogen. Fresh-cut apples usually do not receive sanitizer treatment other than the washing treatments applied to the whole apples before coring and cutting. Studies have reported that human pathogens can grow and reach high numbers without noticeable changes to sensory characteristics of apple products (12). The sectioning method described above allows accurate quantification of the number of the cells at various depths within

punctures and cut surfaces. Future experiments will be needed to examine the importance of deep penetration of bacteria within apple tissues as yet another obstacle to effective decontamination of apples.

The calyx cavity of the apple has been suggested as the region with greatest potential for contamination and a possible route for bacterial entry within the calyx and the ventral cavity of the apple (5, 6, 21). Buchanan et al. (5) observed that differences in the extent of channel opening contributed to dye uptake within the inner core of the apple. In our study, apples with sepals that remained upright or partially upright (open calyx) allowed greater uptake of the inoculum than those with closed calyces, regardless of the method of inoculation. The inoculation procedures used in the calyx studies represent two ways apples can become contaminated. Dip inoculation simulated contamination in which a large portion of the surface may be exposed to the contamination source, e.g., drench or flume water, whereas spot inoculation simulated localized contamination, e.g., from a stem puncture or area of decay. Perhaps because of greater hydrostatic pressure and volume, dip inoculation resulted in a more uniform and slightly deeper infiltration of the bacteria than spot inoculation, especially in the case of the open calyx. Spot inoculation represents a more likely harvest or preharvest type of contamination. Inoculum solutions may travel down the walls of the open calyx cavity and deposit cells along the way, resulting in significantly higher concentration of cells within the cavity as compared with the basin. However, E. coli O157:H7 was not recovered from the ventral cavity or the seed locules at any point throughout our study. Our results contrast with those of other studies that reported penetration of the inoculum within the inner core and seed locules (5, 6, 21). This may have been due to variations in the variety, source, extent of storage of the apple (22), and the method of sample preparation.

Penetration of the NaI solution could be viewed as an indicator of how far sanitizer treatments can penetrate within various regions of the apple. The morphology of the apple calyx and treatment time must be considered when applying sanitizer treatments to inactivate human pathogens on apples. Considering that the reported sanitizer treatment times for apples vary between several seconds and several minutes, there simply may not be enough residence time for the sanitizer to reach the cells deep within the apple. The hydrophobic nature of the apple surfaces will form a barrier for the water. Use of low infiltration pressure (5 lb/in²) of sanitizer solutions may help overcome barriers such as closed calyx as evidenced by CT scan imaging. However, pressurization may also lead to undesirable textural changes to the apple and potential relocation of the contaminants deeper within the apple tissue if they resist the sanitizer. Sectioning the calyx allowed us to distinguish between the concentration of cells that remained outside of the calyx and those that penetrated within the calyx cavity. Using this approach, we can better examine the effects of various treatments in the calyx as well as other regions of the apple. Different methods of inoculation employed in this study can influence effectiveness of sanitizer treatments. Differ-

ences in efficacy of sanitizer treatments as a consequence of method of inoculation have been suggested (17, 18).

The U.S. Food and Drug Administration's Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables suggests cleaning produce to remove as much dirt and debris as possible and sanitizing it to destroy harmful microorganisms (2). The first and most effective line of defense in produce safety is prevention of contamination through good agricultural and manufacturing practices. In order to better estimate the efficacy of a sanitizing treatment, the concentration of microorganisms before and after treatment at various depths within produce needs to be evaluated. In this study, a new method was used to prevent cross contamination during sampling and, thus, yield more accurate data on microbial concentrations at various depths. Furthermore, CT scan imaging was used as a tool to examine penetration of fluid that may represent the treatment solutions and the role of morphological differences in allowing solution uptake within apples. Morphological differences in apple calyces may influence both how far bacteria are able to penetrate within the cavity and whether sanitizing treatments penetrate and make contact with these bacteria. Understanding the modes of contamination and why most decontamination treatments are ineffective may lead to development of more effective strategies that will enhance the safety of fresh horticultural products.

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